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Citation for published version (APA):

Theelen, T. L., Lappalainen, J. P., Sluimer, J. C., Gurzeler, E., Cleutjens, J. P., Gijbels, M. J., Biessen, E. A. L., Daemen, M. J. A. P., Alitalo, K., & Yla-Herttuala, S. (2015). Angiopoietin-2 blocking antibodies reduce early atherosclerotic plaque development in mice. *Atherosclerosis*, 241(2), 297-304. <https://doi.org/10.1016/j.atherosclerosis.2015.05.018>

Document status and date:

Published: 01/08/2015

DOI:

[10.1016/j.atherosclerosis.2015.05.018](https://doi.org/10.1016/j.atherosclerosis.2015.05.018)

Document Version:

Publisher's PDF, also known as Version of record

Document license:

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Angiopietin-2 blocking antibodies reduce early atherosclerotic plaque development in mice



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ARTICLE INFO

Article history:

Received 19 February 2015

Received in revised form

4 May 2015

Accepted 20 May 2015

Available online 3 June 2015

Keywords:

Angiogenesis

Microvascular leakage

Plaque stability

Angiopietin-2

Atherosclerosis

ABSTRACT

Objective: Angiopietin-2 (Ang-2) blocking agents are currently undergoing clinical trials for use in cancer treatment. Ang-2 has also been associated with rupture-prone atherosclerotic plaques in humans, suggesting a role for Ang-2 in plaque stability. Despite the availability of Ang-2 blocking agents, their clinical use is still lacking. Our aim was to establish if Ang-2 has a role in atheroma development and in the transition of subclinical to clinically relevant atherosclerosis. We investigated the effect of antibody-mediated Ang-2 blockage on atherogenesis after in a mouse model of atherosclerosis.

Methods: Hypercholesterolemic (low-density lipoprotein receptor^{-/-} apolipoprotein B^{100/100}) mice were subjected to high-cholesterol diet for eight weeks, one group with and one group without Ang-2 blocking antibody treatment during weeks 4–8. To enhance plaque development, a peri-adventitial collar was placed around the carotid arteries at the start of antibody treatment. Aortic root, carotid arteries and brachiocephalic arteries were analyzed to evaluate the effect of Ang-2 blockage on atherosclerotic plaque size and stable plaque characteristics.

Results: Anti-Ang-2 treatment reduced the size of fatty streaks in the brachiocephalic artery (–72%, $p < 0.05$). In addition, antibody-mediated Ang-2 blockage reduced plasma triglycerides (–27%, $p < 0.05$). In contrast, Ang-2 blockage did not have any effect on the size or composition (collagen content, macrophage percentage, adventitial microvessel density) of pre-existing plaques in the aortic root or collar-induced plaques in the carotid artery.

Conclusions: Ang-2 blockage was beneficial as it decreased fatty streak formation and plasma triglyceride levels, but had no adverse effect on pre-existing atherosclerosis in hypercholesterolemic mice.

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Abbreviations: Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; ApoB, apolipoprotein B; LDLr, low density lipoprotein receptor; MMP, matrix metalloproteinase; VEGF-A, vascular endothelial growth factor-A.

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1. Introduction

Increased plasma levels of the vascular growth factor angiopoietin-2 (Ang-2) occur in several diseases, including cancer [1], systemic infections [2], and in cardiovascular diseases such as acute myocardial infarction [3]. Ang-2 is also increased in highly vascularized, rupture-prone human atherosclerotic plaques [4,5]. Despite the detrimental role of Ang-2 in atherosclerosis suggested by these results, Ang-2 overexpression decreased plaque size in a

mouse model of atherosclerosis [6]. However, we considered that systemic Ang-2 overexpression fails to mimic the function of endogenous Ang-2 in atherogenesis.

Despite increased Ang-2 levels in clinically relevant atherosclerosis and the availability of Ang-2 blocking agents, there have been no attempts to study Ang-2 blockage in cardiovascular diseases. Therefore, we considered it important to study the role of Ang-2 blocking as a therapeutic strategy in atherosclerosis. Because the blocking of vascular endothelial growth factor in cancer therapy increased the risk of unpredicted cardiovascular side effects, such as arterial thrombosis, heart failure or cardiomyopathy [7–10], and because anti-Ang-2 therapy is currently used for cancer treatment as well, we aimed to evaluate the potential cardiovascular risk of anti-Ang-2 treatment in an experimental setting of pre-existing atherosclerosis. In order to investigate the role of endogenous Ang-2, a blocking strategy was adopted. Currently, a wide variety of Ang-2 blocking agents are being tested in clinical trials (some of them in Phase III) against different types of cancer (reviewed in [11]). We used an antibody that inhibits tumor growth in mice by blocking Ang-2 binding to its receptor Tie-2 [12].

The therapeutic potential of Ang-2 blockage is based on the adverse effects of Ang-2 on processes leading to plaque instability: microvessel growth (angiogenesis), decreased pericyte coating of microvasculature and increased vascular permeability [2,13,14], that promotes leukocyte recruitment [13,15,16]. In addition, Tie-2 expressing macrophages respond to Ang-2 by secreting TNF α [17], which can contribute to the inflammation in the vessel wall.

Microvessel growth and permeability, and inflammation are also involved in the transition of a clinically silent, stable atherosclerotic plaque into a vulnerable plaque at risk of fibrous cap rupture and ensuing clinical events [18]. The abundance and compromised structural integrity of plaque microvessels are detrimental to plaque stability, increasing the likelihood of plaque rupture [18–20]. Structurally abnormal microvessels involve the risk of plaque hemorrhage and lead to increased lipoprotein deposition and influx of leukocytes, triggering a pro-inflammatory cycle that results in plaque weakening.

In this study, we investigated the effects of the Ang-2 blocking antibody on fatty streak formation and atherosclerotic plaque progression in aortic root, brachiocephalic arteries and in collar-induced carotid atherosclerosis in LDLR^{-/-} ApoB^{100/100} mice.

2. Materials and methods

2.1. Experimental animals

Ten to twelve week old male LDLR^{-/-} ApoB^{100/100} mice (n = 26) were placed on a high cholesterol diet (42% of calories from fat and 0.15% from cholesterol, no sodium cholate; TD 88173 Harlan Teklad, Boxmeer, NL) for 8 weeks. After 3 weeks of diet, mice underwent collar placement surgery as previously described [21]. In brief, the mice were anaesthetized with isoflurane (induction: 4.5% isoflurane, 450 ml air, maintenance: 2.0% isoflurane, 200 ml air; Baxter International, Helsinki, Finland) and injected with 0.1 mg Rimadyl s.c. (Pfizer, Helsinki, FI). Anesthesia was controlled regularly by visual inspection and toe pinch reflex. Carotid arteries were accessed via a sagittal anterior neck incision and dissected from the surrounding tissue without damaging the vagus nerve or the carotid arteries. A 2 mm silastic collar was placed bilaterally, right under the bifurcation and fixed with three circumferential surgical silk knots.

One day after collar placement surgery, the mice were distributed randomly and injected intraperitoneally with either a monoclonal Ang-2 blocking antibody [12] (n = 14) or a control immunoglobulin (n = 12; IgG) at a dose of 10 mg/kg, as previously

described [22]. The injections were repeated three times weekly over a total time of five weeks.

Food and water were provided *ad libitum* during the entire study. All animal experiments were approved by National Experimental Animal Board of Finland and carried out in accordance with guidelines of the Finnish Act on Animal Experimentation.

2.2. Plasma Ang-2 levels

Plasma Ang-2 concentrations were measured using enzyme linked immunosorbent assay for murine Ang-2 (R&D systems, Abingdon, UK).

2.3. Echocardiography and carotid artery ultrasound

Echocardiographic measurements were performed before the collar operations and at 5 weeks after the operation/antibody treatment using the Vevo[®] 2100 Ultrasound System (VisualSonics[®], Amsterdam, NL). The animals were anesthetized with isoflurane. The Ejection fraction (EF), fractional shortening (FS), left ventricle (LV) mass, LV diastolic and systolic volume were analyzed from parasternal short axis M-mode measurements. EF was calculated by Vevo2100 software using the Teicholz formula [23]. Carotid strain was measured as percent change in the arterial diameter: (SD – DD)/DD, where SD was the systolic and DD the diastolic CCA diameter. Peak wall shear stress at 1 mm proximal to the collar was calculated by the Poiseuille equation: τ (dyn/cm²) = $4 \times V \times \eta / ID$, where V is the peak systolic flow velocity (cm/s), η is the blood viscosity (taken as 0.035 P), and ID is the maximal lumen diameter of the targeted carotid artery (cm) [24,25].

2.4. Total serum cholesterol and triglycerides

Plasma was separated by centrifugation and stored at –80 °C until further use. Cholesterol was determined using standard enzymatic assays (CHOD-PAP method - Cholesterol FS Ecoline product no. 1 1300 99 90 314, DiaSys, Holzheim, GE), as were triglyceride levels (GPO method - Triglycerides FS Ecoline REF 1 5760 99 90 314 both DiaSys).

2.5. Atherosclerotic plaque quantification and immunohistochemistry

Atherosclerotic plaque development in the murine arterial tree starts in the aortic root, extending to the aortic arch and brachiocephalic trunk and, after an extended time period, developing into the carotid bifurcation [26]. In this study, the mice were fed a high cholesterol diet for 8 weeks and subjected to antibody treatment during the last 5 weeks. The plaques in the brachiocephalic artery consisted mainly of foamy macrophages without an overlying cap or extracellular cholesterol crystals, thus termed fatty streaks. The plaques in the aortic root and carotid arteries were classified as advanced plaques composed of immune cells, smooth muscle cells and a necrotic core, all covered by a fibrous cap. The antibody effect on fatty streak formation could thus be studied in the brachiocephalic artery, while the effect on progression of pre-existing, advanced plaques was studied in the aortic root and carotid arteries with a collar placement, respectively.

Mice were euthanized by CO₂ asphyxiation and blood was collected via the right ventricle for ELISA, total cholesterol- and triglyceride analysis. Remaining blood was cleared by perfusion with 20 ml PBS via the left ventricle. Brachiocephalic arteries, aortic root and right common carotid arteries were excised and immersion fixed in 1% paraformaldehyde overnight.

Tissue samples were paraffin embedded, serially sectioned

(4 μm) and stained with hematoxylin and eosin (HE, Sigma, Zwijndrecht, NL) for quantification of the plaque areas using computerized morphometry (Leica QWin V3, Cambridge, UK). Total plaque area and necrotic core content were obtained by averaging measurements of five representative sections of the brachiocephalic artery, aortic root and right common carotid artery. The necrotic core was defined by cholesterol clefts, lipid droplets and acellular regions. Collagen content was determined from representative sections stained with Sirius Red (Sirius Red + area/plaque area; Sigma). Plaque macrophages and the intra-plaque and adventitial microvessels were quantified using immunohistochemistry for Mac-3 (Mac-3⁺ area/plaque area; BD Pharmingen, Breda, NL) and PCAM-1 (BD Pharmingen, Breda, NL) respectively.

2.6. Statistical analysis

All data are presented as mean \pm SEM. Following Shapiro–Wilk test for normal distribution, the groups were compared with student's t-test or Mann–Whitney rank-sum test. (GraphPad Prism4, La Jolla, CA, USA). A p-value of $p < 0.05$ was considered significant.

3. Results

3.1. Ang-2 blocking decreases plasma triglycerides but not plasma cholesterol levels

The Ang2 antibody used in the study blocks the Ang-2 fibrinogen binding domain that is responsible for receptor binding [12], and this results in increased Ang-2 levels in the circulation [27]. The antibody injections resulted in an almost 30-fold increased plasma Ang-2 concentration after five weeks of treatment (Fig. 1A). An increase in plasma Ang-2 has also been described in humans

treated with the anti-Ang-2 antibody [27]. During five weeks of administration, the antibody treatment did not affect the general health status, including body weight (Fig. 1B), cardiac function (Fig. 1E–J) or shear stress in carotid artery after collar implantation (Fig. 1K and L). However, the treatment led to a 27% decrease in plasma triglycerides (Fig. 1D) without any effects on plasma cholesterol (Fig. 1C).

3.2. Ang-2 blocking decreases fatty streak formation

In order to investigate if Ang-2 blockage affects fatty streak formation, the brachiocephalic artery [28] was analyzed after eight weeks of high-cholesterol diet. Interestingly, fatty streak formation in the brachiocephalic artery was significantly less in mice treated with the anti-Ang-2 antibody than control IgG (Fig. 2A–C). While anti-Ang-2 therapy reduced the percentage of stenosis, the lumen and media size remained unchanged (Fig. 2D–F). Furthermore, Ang-2 blockage did not alter the fatty streak composition: the collagen content and the percentage of Mac-3 positive macrophages in the plaques (Fig. 2G–I and J–L, respectively) remained unaltered. No microvessels were found in the fatty streaks and adventitial microvessel density was not altered by the antibody treatment (Fig. 2M–O and data not shown).

3.3. Existing and advanced plaques are not affected by antibody-mediated Ang-2 blockage

Mice were fed a high-cholesterol diet for three weeks followed by anti-Ang-2 antibody treatment while the diet was continued. This allowed us to analyze the pre-existing plaques in the aortic root in addition to fatty streaks in the brachiocephalic artery. Plaque sizes in aortic roots of mice treated with anti-Ang-2 did not

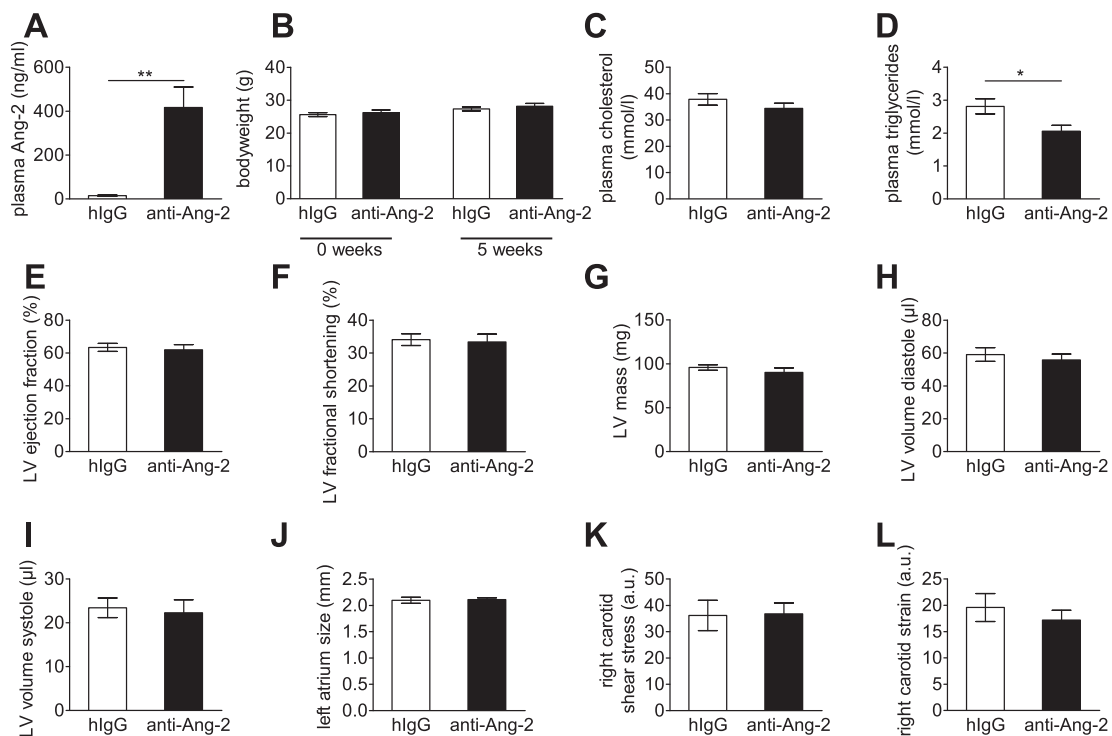


Fig. 1. Anti Ang-2 antibody treatment increases plasma Ang-2 levels and decreases plasma triglycerides. Plasma Ang-2 levels in anti-Ang and IgG treated mice (A). During the five weeks of administration, the antibody treatment did not affect the general health status, body weight (B), cardiac function (E–J) or shear stress in carotid artery after collar implantation (K + L). However, the treatment led to a decrease in plasma triglycerides (D) without effects on plasma cholesterol (C). IgG n = 12; anti-Ang-2 n = 14; * $p < 0.05$; ** $p < 0.01$.

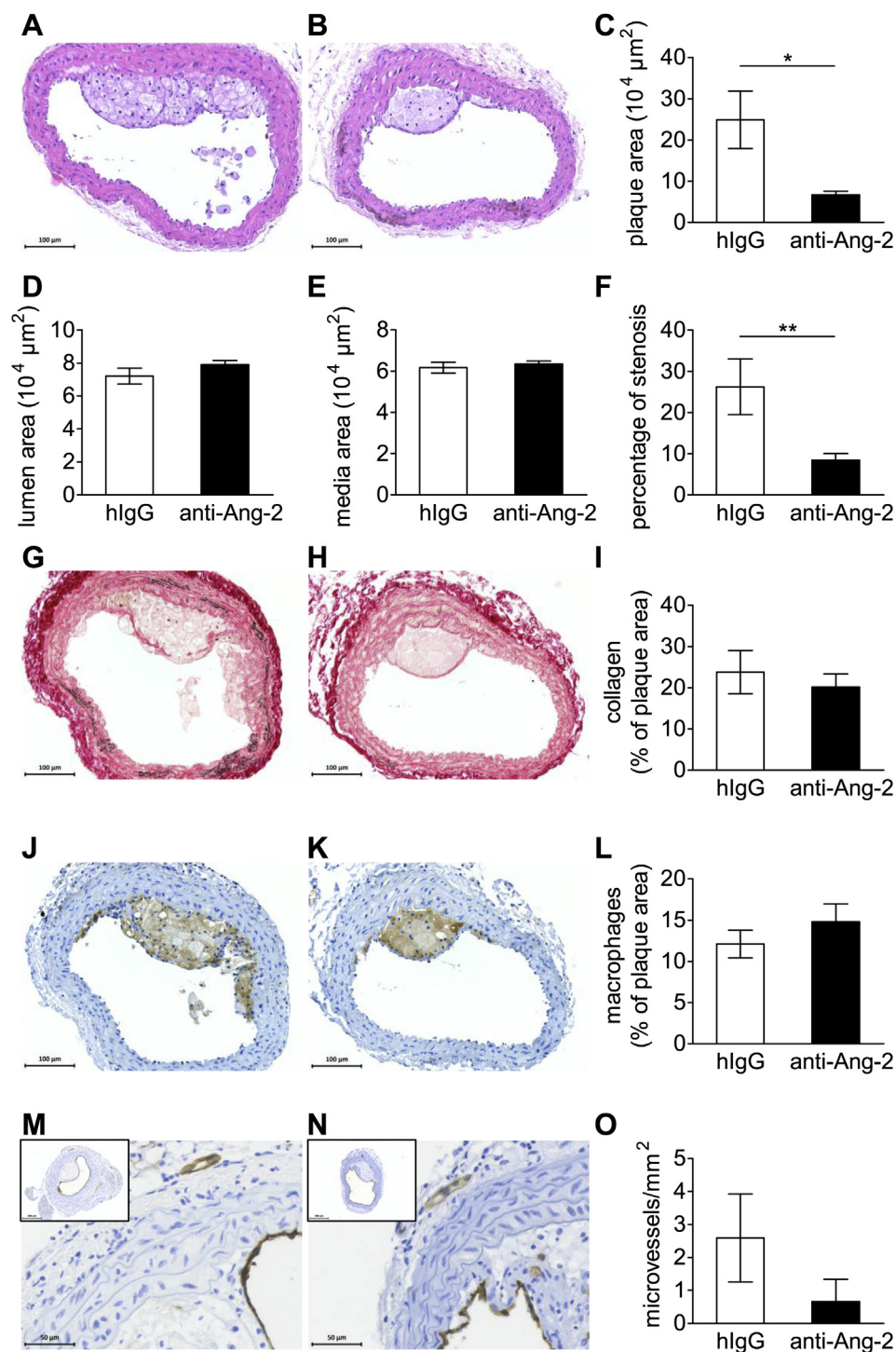


Fig. 2. Antibody mediated Ang-2 blockage delays fatty streak formation in the brachiocephalic artery. Representative images of HE-stained brachiocephalic arteries (A + B) show a decrease in fatty streak size in animals treated with anti Ang-2 antibody (C). There is no difference in lumen area or medial thickness (D + E) but a reduction in stenosis after anti-Ang-2 treatment (F). Plaque collagen content (percentage of Sirius Red positive area/total plaque area, G–I), percentage of macrophage area (Mac3+ area/total plaque area, J–L) and adventitial microvessel density (microvessels/mm²) (MVD) (M–O). Representative images: left panels IgG, right panels anti-Ang-2 antibody. IgG n = 12; anti-Ang-2 n = 14; *p < 0.05.

differ from control IgG injected mice after eight weeks of high-cholesterol diet and five weeks of antibody administration (Fig. 3A–C). In addition, the treatment did not alter the amount of necrotic core (Fig. 3A, B and D), collagen content (Fig. 3E–G) or the macrophages in the plaques (Fig. 3H–J). No changes in intra-plaque vessels or adventitial microvessel density were observed

(Fig. 3K–M and data not shown). Hence, Ang-2 blockage did not interfere with the progression or stability of pre-existing plaques.

In addition to the pre-existing plaques in the aortic root, we were interested in the effects of the antibody on collar-induced fast-growing advanced plaques. The plaque size and necrotic core content in these rapidly progressing collar-induced advanced

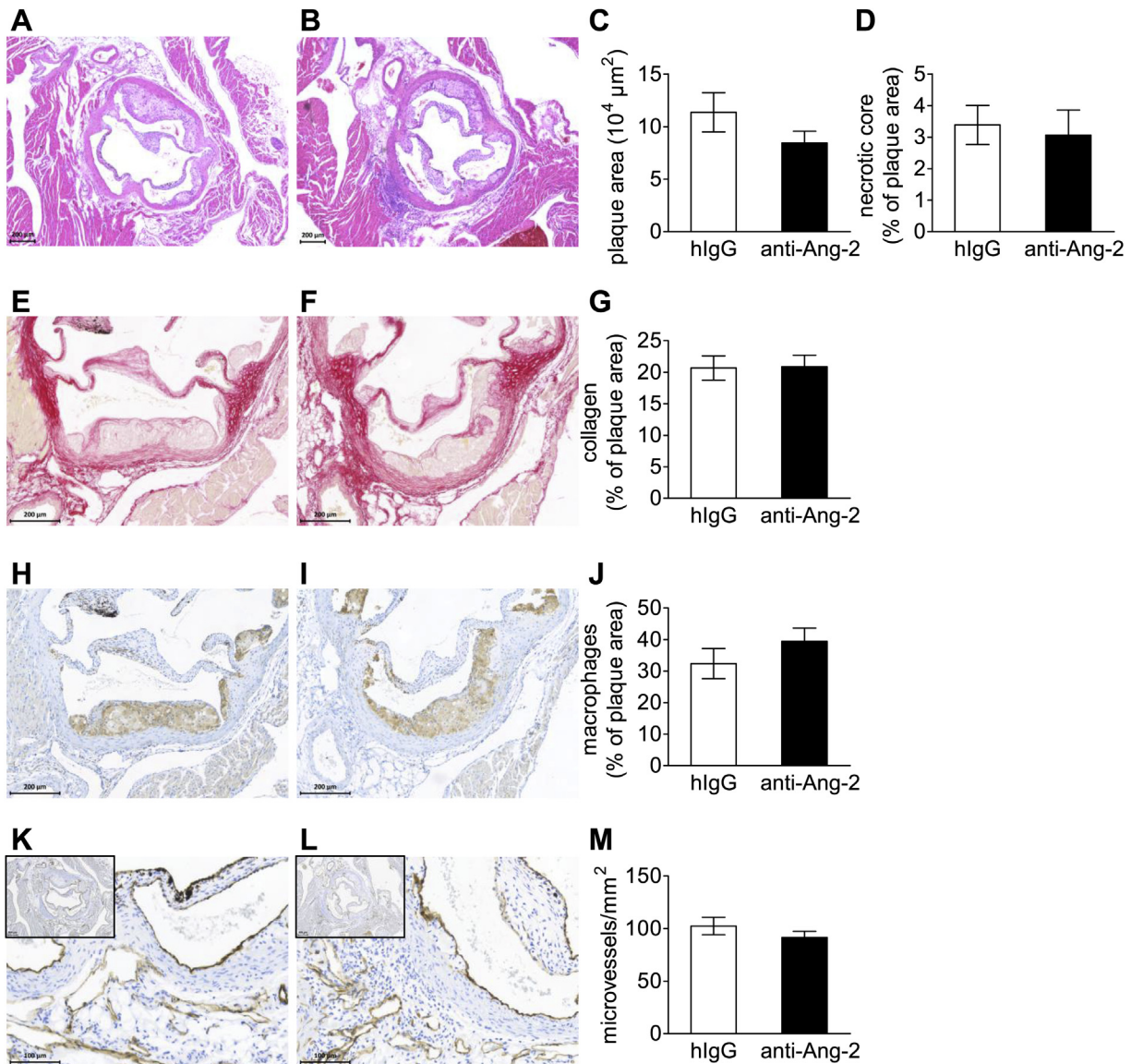


Fig. 3. Anti Ang-2 antibody treatment does not alter pre-existing atherosclerosis in the aortic root. Representative images of HE-stained plaques in the aortic root (A + B) show no difference in plaque size (C) or necrotic core (D) in pre-existing plaques of the aortic root after antibody treatment. Also, collagen (percentage of Sirius Red positive area/total plaque, E–G), macrophage content (Mac3+ area/total plaque area, H–J) and adventitial microvessel density (microvessels/mm², K–M) were not altered by the anti-Ang-2 antibody treatment. Representative pictures: left panels control IgG, right panels anti-Ang-2. IgG n = 12; anti-Ang-2 n = 14.

plaques did not differ between the anti-Ang-2 and control IgG-treated mice (Fig. 4A–D). The effect of anti-Ang-2 on fatty streak formation and atherosclerotic plaque development has been summarized in Table 1.

4. Discussion

Our study is the first one to investigate Ang-2 depletion in experimental atherosclerosis and the results demonstrate that antibody-mediated Ang-2 depletion reduces fatty streak formation in the brachiocephalic arteries. In addition, anti-Ang-2 treatment reduces plasma triglycerides while plasma cholesterol levels remain unchanged. The anti-Ang-2 antibody did not interfere with pre-existing plaques of the aortic root or with collar-induced plaques in the carotid artery. Moreover, Ang-2 depletion had no effect on the stability of the plaque phenotype as microvessel density, macrophage and collagen content and the necrotic cores remained

unchanged (Table 1).

4.1. Ang-2 loss-of-function model contradictory to gain-of-function model in atherosclerosis

To date, inhibition or knock-out of endogenous Ang-2 in human or murine atherosclerosis have not been reported. However, Ang-2 overexpression was shown to decrease plaque formation in the aortic root of ApoE knock-out mice via inhibition of LDL oxidation [6]. However, the impact on plaque stability was not studied in great detail. The authors only observed an absolute decrease in plaque macrophages after Ang-2 overexpression, albeit that data were not corrected for the reduced overall plaque size [6]. Therefore, the reduction in macrophage area probably only reflects the plaque stage. Concordant with this notion, in our study, we did not observe any difference in relative macrophage content (macrophage area normalized to the plaque area) after Ang-2 blockage.

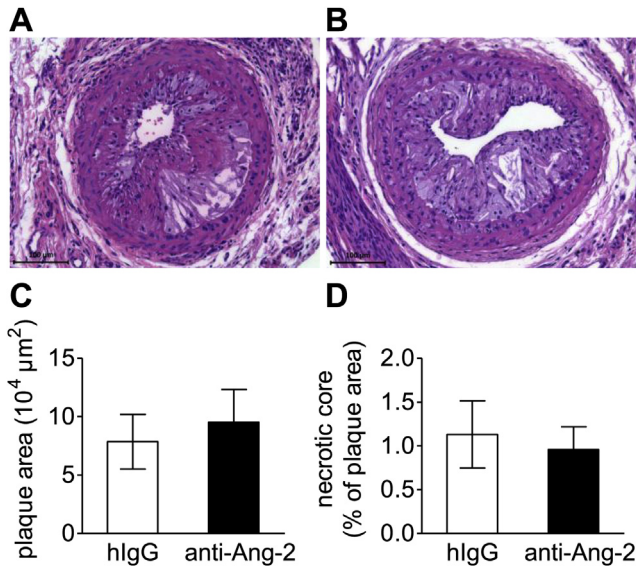


Fig. 4. Advanced lesions in the carotid arteries are not affected by Ang-2 blockage. Representative images of HE-stained collar induced advanced plaques (A + B) in the right common carotid artery. Plaque size (C) necrotic core (D). Representative images and quantification of (E–G) collagen, (H–J) macrophages, (K–M) microvessels: left control IgG, right anti-Ang-2. IgG n = 12; anti-Ang-2 n = 14.

Enhancing Ang-2 may seem an attractive therapy at first sight. However, transgenic Ang-2 overexpression does not mimic the endogenous role of Ang-2 in atherosclerosis, which is crucial for the translation to the human situation.

4.2. No role for Ang-2 blockage in plaque stabilization

Considering the stimulatory role of Ang-2 in various processes associated with vulnerable plaques [18], such as increased inflammation [13], plaque vascularity [5], microvascular leakage [15] and MMP expression [5], we expected profound effects of Ang-2 antagonism on the stable plaque phenotype. However, Ang-2 deletion was only able to prevent fatty streak formation; it did not prevent plaque progression or changes in plaque stability.

Changes in fatty streak formation could not be attributed to effects on plaque inflammation or angiogenesis. Rather, Ang-2 blockage may retard fatty streak formation by a reduction in plasma triglycerides, prevention of endothelial dysfunction or reduction of monocyte chemotaxis. We observed lower plasma triglycerides after Ang-2 antibody treatment, which might explain the decrease in fatty streak formation. Until recently, hypertriglyceridemia was regarded as a risk factor of cardiovascular disease based on epidemiological association only, with much controversy on possible causality. However, recent Mendelian randomization studies increasingly support the causative role for increased plasma triglycerides in cardiovascular disease and atherosclerosis [29,30]. Cellular effects are mediated by remnant lipoproteins, rich in triglycerides, rather than chylomicrons (reviewed in [31]). Mechanistically, direct uptake of these remnant lipoproteins by macrophages stimulates cholesterol ester accumulation and macrophage foam cell formation, an important step in fatty streak formation [32]. Neutral and oxidized free fatty acids generated by lipolysis of these triglyceride-rich lipoproteins can also stimulate endothelial dysfunction, subsequently enhancing vascular inflammation and lipoprotein retention [33], and hence fatty streak formation [31]. Thus, lower triglycerides may prevent endothelial dysfunction and foam cell formation, explaining the reduction in fatty streak formation. Although high triglyceride levels have also been associated with clinically relevant atherosclerosis [34], reduced levels were without effect on pre-existing atherosclerosis in our model.

Ang-2 blockage could decrease fatty streak formation also via processes that are regulated by the angiopoietin receptor Tie2, which is highly expressed in endothelial cells and monocytes, both involved in the early development of atherosclerosis. Ang-2 expressed by endothelial cells has been shown to stimulate monocyte chemotaxis [35], hence Ang-2 blockage may reduce monocyte recruitment to developing fatty streaks. However, as we did not observe alterations in macrophage content of fatty streaks, this seems unlikely. More likely, is the involvement of endothelial cell dysfunction and the subsequent permeability for lipoproteins. This is thought to be the initial step at the onset of atherosclerosis. Ang-2 is known to induce endothelial destabilization and vascular permeability [36], thus suggesting a beneficial effect of Ang-2 blockage.

Table 1

Summarizing table of the described data.

	Parameter	Tissue	Alteration in anti-Ang-2 mice	Statistics
Plaque burden	Plaque size	Brachiocephalic artery	↓	*
		Collar model	=	n.s.
		Aortic root	=	n.s.
Plaque phenotype	Degree of stenosis	Brachiocephalic artery	↓	**
		Brachiocephalic artery	Absent in fatty streak	—
	Necrotic core	Collar model	=	n.s.
		Aortic root	=	n.s.
		Brachiocephalic artery	=	n.s.
	Plaque collagen	Aortic root	=	n.s.
		Brachiocephalic artery	=	n.s.
	Plaque macrophages	Brachiocephalic artery	=	n.s.
		Aortic root	=	n.s.
	Microvessel density	Brachiocephalic artery	=	n.s.
		Aortic root	=	n.s.
Systemic effects	Angiopoietin-2	Plasma	↑	**
	Cholesterol	Plasma	=	n.s.
	Triglycerides	Plasma	↓	*
	Cardiac function	US of the heart	=	n.s.
	Carotid strain + shear stress	US of carotid artery	=	n.s.
	Body weight	Before sacrifice	=	n.s.

US = ultra sound; n.s. = not significant.

*p < 0.05, **p < 0.01.

4.3. Used Ang-2 antibody proven to be effective in other disease models

The Ang-2 blocking capacity of anti-Ang2 antibodies in vitro [12] and in vivo [12,22,27,37] has been firmly established and was confirmed in our study by using Ang-2 ELISA. It has been reported that treatment of cardiac allografts with anti-Ang2 protects from transplant inflammation and rejection [37]. This is in line with atherosclerosis inhibition by overexpression of the Tie2 agonist ligand, Ang1 in cardiac allografts [38]. Antibody-mediated Ang-2 blockage has been proven to reduce tumor growth [12] and lung metastasis formation [22] in murine cancer models by increasing tumor vessel stability. Moreover, Ang-2 is released by endothelial cells [39], thus anti-Ang-2 antibody has perfect access to the target through the adventitial microvasculature and the main arteries. It is thus unlikely that Ang-2 depletion or impaired tissue availability of the antibody could explain the lack of effect on plaque progression and stable plaque phenotype.

4.4. Inhibiting Ang-2 alone might not be sufficient

Alternatively, inhibiting Ang-2 only might not suffice to affect pre-existing atherosclerosis. Additional factors, such as vascular endothelial growth factor A (VEGF-A) should be targeted as well. For example, one could employ a bifunctional anti-VEGF-A/anti-Ang-2 antibody, which combines the inhibition of both growth factors; this has already provided a stronger anti-tumor effect than targeting the two growth-factors separately [40].

4.5. Ang-2 cause or consequence of atherosclerosis

The lack of effect on plaque growth or the stable plaque phenotype is in contrast to previously reported associations between Ang-2 and cardiovascular disease. Increased Ang-2 plasma levels have been correlated with cardiovascular disease progression, but have also been suggested to provide a biomarker for future cardiovascular events such as myocardial infarction or stroke [3,41–47]. Furthermore, Ang-2 was detected in advanced vascularized human plaques [48] and it was associated with a high microvessel content and destabilizing MMP-2 expression [5]. The outcomes of these studies support our findings that Ang-2 depletion helps to inhibit atherosclerosis development. Nevertheless, not all studies could show a correlation of Ang-2 with plaques at risk of rupture [49,50]. Perhaps the clinical correlation of Ang-2 expression and atherosclerosis reflects a compensatory expression in response to plaque vulnerability. Overall, Ang-2 may be correlated with cardiovascular diseases, but the results described in the present work do not support a causal role for it in atherosclerosis progression or plaque vulnerability. Importantly, our study gives no reason to think that Ang-2 blockage could have adverse effects on pre-existing atherosclerosis of treated cancer patients.

5. Conclusions

This is the first time Ang-2 blockage has been tested in an experimental model of atherosclerosis and found to have significant protective effects on fatty streak formation. Ang-2 blockage reduces plasma triglycerides and decreases early plaque formation, suggesting a beneficial effect of Ang-2 depletion on the early phase of atherosclerosis. In addition, no effects on pre-existing atherosclerosis or cardiac function were found, suggesting that anti-Ang-2 therapy is safe in various clinical settings.

6. Significance in the context of atherosclerosis

The present study is the first to investigate the effect of blocking Ang-2 on experimental atherosclerosis. In addition, the cardiovascular safety of the Ang-2 blocking was studied. It was shown that anti-Ang-2 therapy delayed fatty streak formation and decreased plasma triglyceride levels. Pre-existing atherosclerosis and cardiac function were unaltered. The results suggest a favorable safety profile for the clinical use of antibody mediated Ang-2 blockage.

Sources of funding

Research was supported by Finnish Academy (141069), ERC (ERC-2009-AdG-250050, FutureGenes) grant, Sigrid Juselius Foundation, Finnish Foundation for Cardiovascular Research (all to S.Y.); the European Research Council (ERC-2010-AdG-268804, VESSEL network), Leducq Transatlantic Network of Excellence on Lymph Vessels in Obesity and Cardiovascular Disease (11CVD03) (all to K.A.), a VENI fellowship of the Netherlands Organization of Scientific research (to J.C.S. 016.116.017); a PhD-student fellowship from the Cardiovascular Research Institute Maastricht (to T.L.T.).

Disclosures

None.

Acknowledgements

The authors would like to thank Dr. ChingChing Leow and MedImmune for the anti-Ang-2 antibodies, and the staff of the Experimental animal facility at the University of Eastern Finland, Kuopio.

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